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ORIGINAL ARTICLE

Effect of implant cleaning on titanium particle dissolution and cytocompatibility

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Abstract

Background: Peri-implantitis treatments are mainly based on protocols for teeth but have not shown favorable outcomes for implants. The potential role of titanium dissolution products in peri-implantitis necessitate the consideration of material properties in devising treatment protocols. We assessed implant cleaning interventions on (1) bacterial removal from Ti-bound biofilms, (2) Ti surface alterations and related Ti particle dissolution, and (3) cytocompatibility.

Methods: Acid-etched Ti discs were inoculated with human peri-implant plaque biofilms and mechanical antimicrobial interventions were applied on the Ti-bound biofilms for 30 seconds each: (1) rotary nylon brush; (2) Ti brush; (3) water-jet on high and (4) low, and compared to sterile, untreated and Chlorhexidine-treated controls. We assessed colony forming units (CFU) counts, biofilm removal, surface changes via scanning electron microscopy (SEM) and atomic force microscopy (AFM), and Ti dissolution via light microscopy and Inductively-coupled Mass Spectrometry (ICP-MS). Biological effects of Ti particles and surfaces changes were assessed using NIH/3T3 fibroblasts and MG-63 osteoblastic cell lines, respectively.

Results: Sequencing revealed that the human biofilm model supported a diverse biofilm including known peri-implant pathogens. WJ and Nylon brush were most effective in reducing CFU counts ($P < 0.01$ versus control), whereas Chlorhexidine was least effective; biofilm imaging results were confirmatory. Ti brushes led to visible streaks on the treated surfaces, reduced corrosion resistance and increased Ti dissolution over 30 days of material aging as compared to controls, which increase was amplified in the presence of bacteria (all P-val < 0.05). Ti particles exerted cytotoxic effects against fibroblasts, whereas surfaces altered by Ti brushes exhibited reduced osteoconductivity versus controls ($P < 0.05$).

Conclusions: Present findings support that mechanical treatment strategies selected for implant biofilm removal may lead to Ti dissolution. Ti dissolution should become an important consideration in the clinical selection of peri-implantitis treatments and a necessary criterion for the regulatory approval of instruments for implant hygiene.

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KEYWORDS
biofilms, cellular microenvironment, corrosion, dental implants, peri-implantitis, surface properties, titanium

1 | INTRODUCTION

Peri-implantitis is a prevalent disease with limited effective treatment strategies.[1] Its etiology is considered to be akin to that of periodontitis,[2] therefore existing peri-implantitis treatments target titanium-bound biofilms. However, these treatments have little to no medium-term effects.[3,4] Because antimicrobial treatments are favored, peri-implantitis research is mainly directed towards identifying applicable methods for implant surface detoxification.[5-7] However, the application of antimicrobial agents on implants may have adverse effects on physicochemical characteristics of the decontaminated surfaces.[8] Chemotherapeutic agents that are clinically employed to reduce peri-implant bacterial burden are adsorbed by Ti surfaces and, as in the case of chlorhexidine (CHX), may adversely compromise cellular re-attachment to the implant surface.[5,8-10] Less is known regarding mechanical cleaning aids and instruments used for peri-implant plaque biofilm removal in the context of prevention, or peri-implantitis therapy. The available information supports that mechanical aids may cause surface alterations, but the effects of the alterations on clinical disease remain largely unknown.[11]

The finding that antimicrobial implant treatment interventions may in fact compromise the cytocompatibility of implant surfaces has raised awareness of the need to characterize implant surface effects in addition to bacterial removal efficacy. A major step forward towards the realization of the role of Ti degradation products in peri-implant inflammation was recently made by Eger et al.[12] who found that ultrasonic scaling around implants, the most frequent means of teeth cleaning by dentists and hygienists, led to the release of Ti microparticles in vitro. Importantly, these microparticles induced expression of pro-inflammatory cytokines by macrophages *in vitro* and resulted in osteolysis when implanted in mouse calvariae in vivo.[12] Similarly, Heyman et al. showed an effect of Ti on impaired differentiation of Langerhans cells in murine epithelium, which was linked to Ti ions in vitro.[13] These findings are important in that they raise vigilance regarding the potential adverse events related to the use of mechanical treatments for implant surface cleaning. There is a need to perform an in-depth investigation of the suitability of existing mechanical treatments around implants, because there is currently no clear guideline on how to perform preventive cleaning of dental implants or detoxify implant surfaces in the context of peri-implantitis.[1] Nonetheless, there is a critical gap in knowledge because existing studies have only assessed Ti wear particles that are generated by direct mechanical abrasion of Ti implants.[12] The long-term effects of implant surface damage are not well-studied. Ti alloys are suitable as implantable biomedical devices because they resist dissolution in biological fluids even under extreme pH levels and under the presence of oxidizing agents, such as Reactive Oxygen Species (ROS). Although direct mechanical damage, such as use of ultrasonic or implantoplasty generate wear particles, the long-term effects of implant surface damage are not well studied.[14] Importantly, the consistent finding of vastly increased Titanium Dissolution Products (TiDissPs) in peri-implant tissues[15] and plaque biofilms [16] strongly suggests a long-term dissolution effect of Ti in peri-implant disease that needs to be investigated. Dissolution products arise when a compound forms a solution over time and Ti dissolution testing focuses on the extent and rate of Ti solution formation. If long-term Ti dissolution is triggered as a result of implant cleaning, the ramifications could be major. Ti particles participate in activation of leukocytes[17] leading to the secretion of superoxide anions, which cause damage to peri-implant tissue cells, and IL-1b secretion with major pro-inflammatory effects.[18-20]

The epidemiological findings of TiDissPs concentration increases being associated with in peri-implantitits has recently led clinical researchers to challenge the removal of bacterial biofilms as being sufficient for the resolution of peri-implant inflammation.[21] To this extent recent investigations of peri-implantitis as a multifactorial entity have established a strong association between TiDissPs and prevalent peri-implantitis.[16,22,23] Olmedo et al. were the first to document the presence of TiDissPs in the peri-implant tissues via exfoliative cytology.[15] Specifically, Ti was observed both inside and outside epithelial cells and macrophages.[15] TiDissPs concentrations were increased under inflammatory conditions as compared to relative clinical health, which was suggestive of their role in peri-implant inflammation. Subsequently, Safioti et al.[16] identified the presence of TiDissPs in peri-implant plaque, which further highlighted the complex dynamics among biomaterial surfaces, oral biofilms and host immunity that define the peri-implant milieu.[24] The implication of TiDissPs in peri-implantitis defines a critical juncture

in the peri-implantitis literature because it represents a distinct biomaterial-related factor that may participate in the initiation of and/or progression of peri-implant inflammatory bone loss. Nonetheless, current investigations on the effects of mechanical implant cleaning interventions do not assess implant surface changes that may lead to TiDissPs. Thus, the potential adverse effects of treatments remain unknown. To this end, we sought to determine the effects that mechanical biofilm removal as part of peri-implantitis treatments has on Ti implant surfaces, and whether surface changes are associated with increases in Ti dissolution and loss of cytocompatibility.

2 | MATERIALS AND METHODS

2.1 | Peri-implantitis biofilm model

To test our hypotheses while ascertaining a high translational potential we used a clinically relevant peri-implantitis model, which employs acid-etched microrough Ti grade V surfaces as substrates and multi species human plaque biofilms, as previously described.[8,10] In the present investigation we further improved retention from clinical samples using SHI medium for biofilm growth, which combines the ingredients of several selected media that can support different subpopulations from direct clinical oral samples to better sustain the original oral microbial diversity.[25] Biofilm composition was assessed via shotgun metagenomic sequencing using an Illumina MiSeq[*] platform and relative taxonomic abundance was attained using Metaphlan (metagenomic phylogenetic analysis).[26] We confirmed the ability of this medium to support a diverse community of oral taxa known to be associated to human peri-implantitis via metagenomics sequencing (Figure S1 in online *Journal of Periodontology*). Information on the growth conditions and metagenomics pipeline can be found in the supplemental methods in online *Journal of Periodontology*.

2.2 | Simulation of clinical treatments

Two hundred Ti grade V (Ti-6Al-4V) discs with a diameter of 10 mm were acid etched following a proprietary etching protocol to obtain microrough surfaces identical to commercial dental implants.[†] Prior to experiments, surface contaminants were removed by ultrasonication in cyclohexane followed by washing in acetone and ethanol and DI water. Discs were sterilized via autoclaving for 15 minutes at 121°C. Depending on the assays, sterile acid etched Ti discs, or identical Ti discs with the peri-implantitis biofilm model were treated with clinically relevant dental hygiene treatments and/or chemotherapeutic agents (Figure 1, Table S1 in online *Journal of Periodontology*). For between-experiment reproducibility in the biofilm assays the approximate number of bacteria in liquid culture was standardized via OD600 and incubation times were optimized in pilot time series experiments to consistently yield high-coverage robust biofilms with >95% viability (data not shown). These growth conditions were used consistently for all experimental groups and reproducibility of freeze-thaw cycles was confirmed via metagenomics (see Figure S1 in online *Journal of Periodontology*).

The simulated clinical treatments were selected based on commonly used interventions by practitioners for implant hygiene (see Table S1 in online *Journal of Periodontology*).[27,28] When solutions of Sterile saline (Control) or 0.12% CHX were used as negative and positive controls, respectively, Ti discs were immersed in them for 30 seconds. For mechanical treatments, all interventions were applied for 30 seconds on the Ti surfaces using either a surgical implant motor function at 300 RPMs (Nylon (Ny) brush[‡] and Ti brush[§]) or a pressurized water-jet (WJ) system for oral hygiene[¶] at minimum (Low) or maximum (High) pressure settings. All experiments were performed at least in duplicate.

2.3 | Antimicrobial treatment of Ti surfaces with mechanical treatments

To assess whether mechanical interventions have antimicrobial effects we performed colony forming units (CFU) counts following interventions. Briefly, following biofilm growth and application of the interventions, the treated discs were washed and then sonicated in 1 mL of PBS with 0.5% TWEEN for 30 seconds. The obtained biofilm samples were diluted to 10^4 and 10^5 in sterile PBS and plated onto non-selective sheep blood agar plates. Plates were incubated in an anaerobic chamber for 120 hours and evaluated. Biofilm removal was assessed via LIVE/DEAD staining[#] with 2 μL of 3.34 mM SYTO 9 and of 3.34 mm propidium iodide per mL, incubated in the dark and evaluated under confocal microscopy.

[*] Illumina, San Diego, CA.
[†] Zuga Medical, Chagrin Falls, OH.
[‡] prototype brush.
[§] Straumann, Andover, MA.
[¶] Waterpik Inc., Fort Collins, CO.
[#] LIVE/DEAD BacLight Bacterial Viability Kit, Invitrogen Molecular Probes, Burlington, MA.

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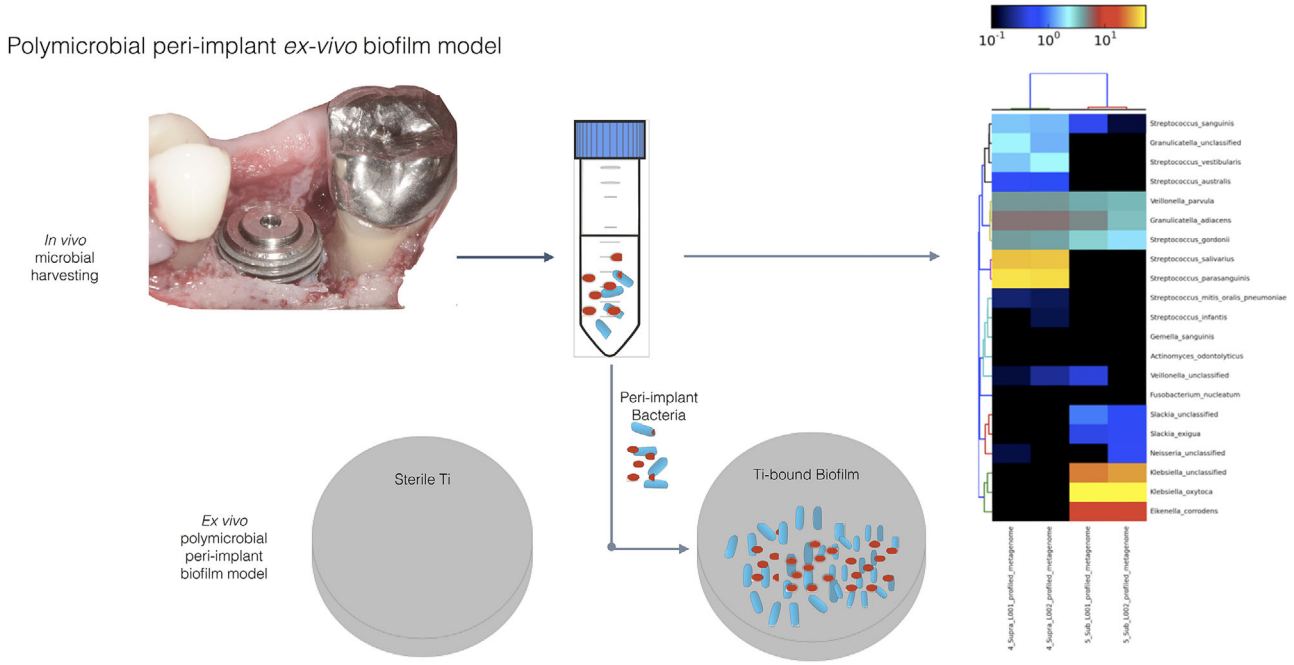

FIGURE 1 Experimental workflow initiated with a human total peri-implant plaque sample, which was used to inoculate acid etched titanium surfaces to form ex vivo Ti-bound biofilms (Note: Figure can be enlarged in online version of article.)

2.4 | Effect of Ti surface changes on corrosion resistance and Ti dissolution rates

To assess surface alterations caused by mechanical peri-implantitis treatments we employed scanning electron microscopy (SEM), atomic force microscopy (AFM) and stereomicroscopic imaging. Further, a previously described electrochemical cell model of peri-implant inflammation was employed to determine mechanical treatment effects on corrosion resistance.[29] Description of the Ti surface survey methods and electrochemical model can be found in the supplemental methods in online *Journal of Periodontology*.

Although it is clinically observed that increased concentrations of TiDissP are associated with peri-implant inflammation it is not known if dental treatment-related surface changes lead to long-term increased Ti dissolution. We assessed whether Ti surface damage by mechanical interventions leads to increased Ti dissolution by assessing TiDissP dissolution after 30 days of material aging of treated samples. To eliminate wear particle effects and focus on long-term TiDissPs only, we eliminated any titanium particles generated as direct effects of the mechanical interventions by repeated rinsing with PBS (×3) and transfer to fresh glass vials to avoid Ti particle entrapment in plastic. Samples were immersed in a protein-rich electrolyte solution simulating tissue fluids (MEM and 10% FBS§) either in ambient (aerobic) or anaerobic conditions for 30 days simulate Ti degradation during aging.[30] TiDissP concentrations were determined at endpoint via Inductively-coupled Mass Spectrometry (ICP-MS) as previously described,[16] or assessed in solution via transmitted light microscopy.

2.5 | Cellular responses to Ti

To determine the biological effects of TiDissPs, a cytotoxicity model employing NIH/3T3 murine fibroblasts was used. Cells were seeded on lysin-coated coverslips in six-well plates at a density of 20,000 cells/well and grown in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin antibiotics (DMEM+) at 37°C in a humidified atmosphere of 5% CO_2. Upon reaching 80% confluency the cells were stimulated with TiDissPs generated as described above for 24 hours. Viability was then assessed after staining with Fluorescein diacetate* and Propidium iodide‡‡ that stain live (green) and dead (red) cells, respectively. Following incubation in the dark, the coverslips were mounted onto glass slides with antifading agent and imaged under confocal microscopy.

To determine effects of Ti surface changes on their cytocompatibility, we used an in vitro model simulating

* Live/Dead Cell Double staining kit, MilliPoreSigma, Burlington, MA.

re-osseointegration of treated implant surfaces. Clinical re-osseointegration is contingent upon the ability of osteoblasts to attach on previously biofilm-contaminated implant surfaces and establish new bone-to-implant contact.[5] Because it has been previously shown that that peri-implantitis treatments affect osteoblastic attachment and proliferation but not differentiation on the treated Ti surface,[8] we employed an established a previously established in vitro re-osseointegration model to determine the ability of osteoblastic cells to proliferate on previously contaminated Ti discs following interventions.[8,10] Briefly, Ti discs were coated with peri-implant biofilms as described above and interventions were applied for biofilm removal (Table S1 in online *Journal of Periodontology*). MG-63 cells were then seeded on the experimental and sterile control discs at a density of 5000 cells/cm^2. The cells were cultured for 3 days in DMEM+ and grown as described above. On the third day, the experimental disks were stained with Fluorescein diacetate and Propidium iodide and imaged in an inverted fluorescence microscope. Three regions of interest per disc were imaged and cell counts were performed. All experiments were performed with cells of the fourth-fifth passage and in triplicate.

2.6 | Statistical analysis

Descriptive analysis were performed using means and standard deviations. Inter-group comparisons were performed via ANOVA followed by Tukey's post-hoc tests to adjust for multiple comparisons.

3 | RESULTS

3.1 | Peri-implantitis biofilm model composition

Metagenomic sequencing revealed reproducibility of biofilms grown at different runs from the same plaque inoculum. Biofilms grown from submucosal plaque samples retained 40 oral taxa and had a distinct microbial signature enriched in gram negative oral taxa as compared to supramucosal plaque controls from the same subject (Figure S1 in online *Journal of Periodontology*), which validated the sampling strategy. The composition of the biofilms formed on the experimental discs were rich in oral taxa known to be prevalent in peri-implantitis, such as S. *sanguinis*, *V. parvula* and *Neisseria* spp. (Figure S1 in online *Journal of Periodontology*).

3.2 | Antimicrobial effects of dental treatments on Ti

In positive controls treated with saline, bacterial colonies exhibited nearly confluence, which was in agreement with live/dead fluorescence microscopy showing abundant dense areas of strong green positive live cells (Figure 2). In negative controls, 0.12% CHX-treated samples demonstrated several colonies, but reduced in numbers as compared to positive controls. Biofilm imaging demonstrated residual biofilm in the CHX group with live and dead bacteria signifying that cells are being killed but not removed, consistent with known moderate effects of CHX against Ti-bound biofilms. Mechanical treatments had varying antibacterial effects ranging from a minor non-significant CFU reduction with the Ti brush versus control ($P > 0.05$) to near complete elimination with only one residual colony in WJ ($P < 0.01$) (Figure 2). Efficacy of biofilm removal mirrored CFU results for the WJ application, which led to near complete (>90%) biofilm removal for both high and low settings (Figure 2). The Ny brush also had an antimicrobial effect with significantly less CFUs as compared to control ($P < 0.01$) (Figure 2).

3.3 | Surface alterations

To assess Ti surface damage as an adverse event of mechanical peri-implantitis treatments, we assessed surface morphology via AFM and SEM. A 30 seconds application of a Ti brush using a rotating surgical implant handpiece at 300 RPM led to mechanical surface alterations of the Ti surfaces with the streaks of the rotating Ti Brush being visible under AFM as "valleys" and pits (Figure 3, Figure S2 in online *Journal of Periodontology*). These changes were linked to the generation of Ti wear microparticles as a direct effect of cleaning (Figure 3). Corresponding SEM scans depicted partial biofilm removal consistent with intermittent contact of the brush with the surface. Ny Brush and WJ treated surfaces were the least aggressive intervention with surface patterns similar to control group consistent with no surface Ti mechanical abrasion (Figure S2 in online *Journal of Periodontology*).

3.4 | Reduced corrosion resistance and increased Ti dissolution rates

To determine corrosion resistance we first assessed the passive tendency of the treated samples to participate in electrochemical corrosion in biological fluids by measuring Open circuit potential (OCP) values. Stability of the

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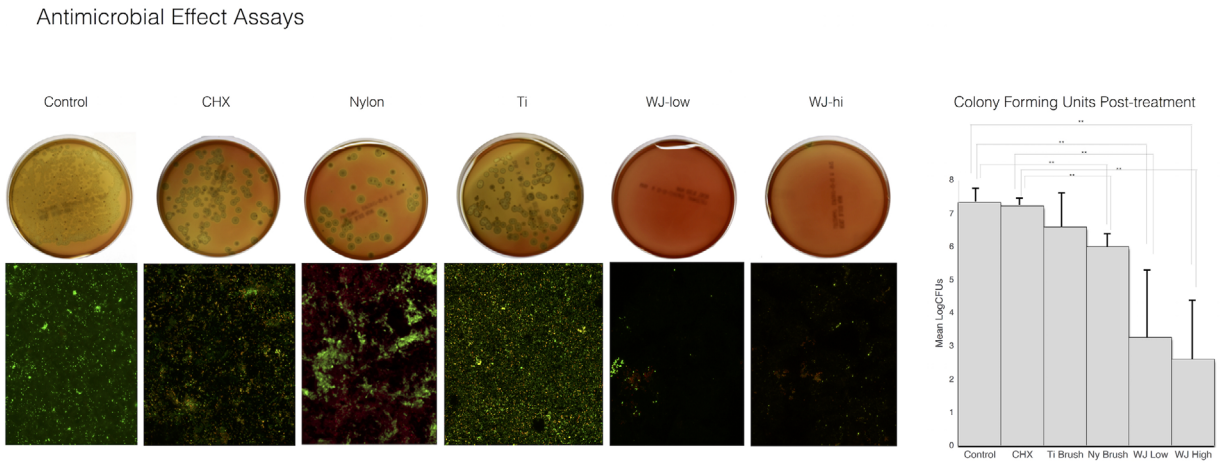

FIGURE 2 Antimicrobial effects of dental treatments on titanium (Ti). Upper row: representative colony forming units (CFU) results on sheep blood agar plates from each group showing antibacterial effect in descending order for WJ High > WJ Low > Nylon > CHX > Ti Brush > Control. WJ groups outperformed the remaining interventions with only 1 CFU present in the WJ low group and none in the high group. Lower row: live/Dead fluorescence biofilm imaging mirrored results from the CFU counts except for case of chlorhexidine (CHX) where the reduction in CFUs as compared to control was not associated with biofilm removal; the majority of dead bacteria remained adherent on the Ti surface

samples was found to be dependent on the intervention-induced surface alterations and the residual biofilm. For the Nylon brush and WJ groups, OCP values were -0.1 V at baseline and stabilized after few minutes of immersion. For the Ti Brush, the baseline OCP was more electronegative (-0.15 V) and exhibited the longest lag observed until stabilization after ~28 minutes (Figure 4). Biofilm-coated untreated samples served as positive controls, because the organic biofilm coating is known to impede electrical conductivity. Correspondingly, the untreated control demonstrated the largest baseline impedance with the potential largely increasing with the time of immersion to approximately + 0.18V. Organic surface films act as a dielectric, because they present low electrical conductivity properties.[31] Thus, OCP results have to be interpreted in relation to the residual biofilm on each surface, because the carbon rich residuals biofilm may interpose as a barrier between the corrosive medium and the metallic material. After stabilization, samples treated with pressurized saline (WJ High) exhibited the most stable behavior among all treatments groups (-0.09V), whereas the lowest OCP potential, that is, most unstable surface with highest corrosive dissolution potential, was found in the samples treated with the Ti brush (-0.15V). Notably, despite the presence of more electrochemically resistant residual biofilm in the TiBr-treated group as compared to the WJ-treated group, the former exhibited the most negative OCP values. This finding is consistent with the aforementioned grooves and streaks observed on the Ti surfaced caused by the Ti brush, as shown in Figure 3. These areas of mechanical damage appeared differentially colored by backscatter electrons under SEM imaging with strong Ti signals (EDX data; Figure 3). Electrochemical impedance spectroscopy (EIS) was also employed to assess electrochemical corrosion dynamics. EIS results mirrored these of OCP assessments with lowest in the Nyquist diagrams shown for the Ti brush group, whereas WJ demonstrated the highest impedances among intervention groups (See Figure S3 in online *Journal of Periodontology*). The WJ and Ny brush groups exhibited Bode phase angle diagrams closest to the positive control (See Figure S3 in online *Journal of Periodontology*).

These differences in corrosion resistance were linked to changes in dissolution rates that differed depending on type of mechanical treatment applied. When treated surfaces were aged in the absence of bacteria, Ti concentrations after 30 days of biological aging were significantly higher for Ti brush as compared to sterile controls ($P < 0.05$) (Figure 4). WJ and Nylon brush treated groups exhibited dissolution comparable to that of controls ($P > 0.05$). Results were more dramatic for Ti samples inoculated with peri-implant biofilms as compared to sterile samples. TiDissPs increased for all groups independent of intervention when aged in the presence of bacteria. Ti brush-treated samples exhibited the highest Ti dissolution, which was over two-fold increased as compared to the same intervention applied on sterile Ti samples ($P < 0.05$). Untreated, biofilm-covered discs also exhibited significantly higher Ti dissolution after 30 days as compared to sterile controls ($P < 0.05$), consistent with the effect of peri-implant biofilms on what has been previously referred to as "biocorrosion."

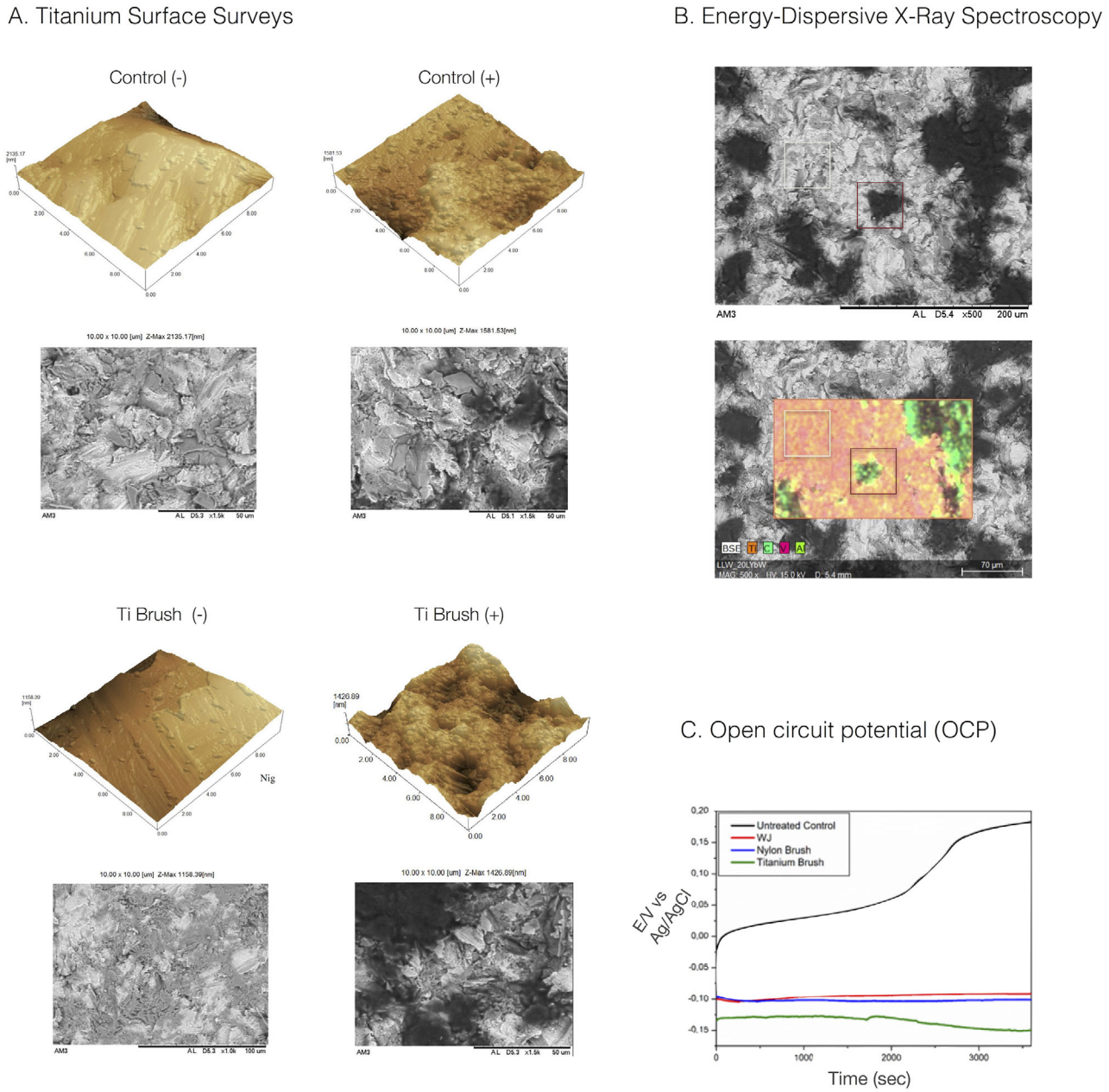

FIGURE 3 (**A**) Atomic force microscopy (AFM) surveys examining effects of mechanical intervention (titanium (Ti)Brush) on sterile [Control (-)] and biofilm-inoculated [Control (+)] Ti implant surfaces. Bacterial cell bodies are evident in Control (+) surfaces, whereas the effect of Ti brush treatment is shown as having a characteristic flattening of the Ti surface with nearly 50% reduced peaks on the *z*-axis in the Ti Brush (-) group and visible strokes across the biofilm covered area with "patchy" biofilm removal in the Ti Brush (+) group. Scanning electron microscopy (SEM) microphotographs after material aging reveal granular corrosion in the areas of mechanical abrasion in Ti Brush (-). (**B**) EDS elemental composition analysis confirms carbon presence in depicted dark residual biofilm regions and strong Ti signals (red square) in areas of backscatter electron exposure consistent with surface alterations (white rectangle); orange: Ti, green: carbon. The dark area inside the black box appears green following the evaluation, indicating that this region is dark because of residual organic biofilm. Conversely, the grey-black area in the white circle appear as red-orange, indicating Ti and V present on the surface, an indication of corrosion. (**C**) Open circuit potential variation with time exhibits a distinct difference in impedance among the Ti Brush and the remaining interventions

3.5 | Effect of Ti surface changes on cell proliferation

Significant cytotoxic effects were observed following the 24 hours stimulation of murine fibroblasts with a 50 ppm concentration of aged TiDissPs, showing a 15% increase in dead cells in the Ti stimulation group versus control ($P < 0.05$) (Figure 5).

In the osteoblastic proliferation model, dental treatments were invariably linked to reduced cytocompatibility

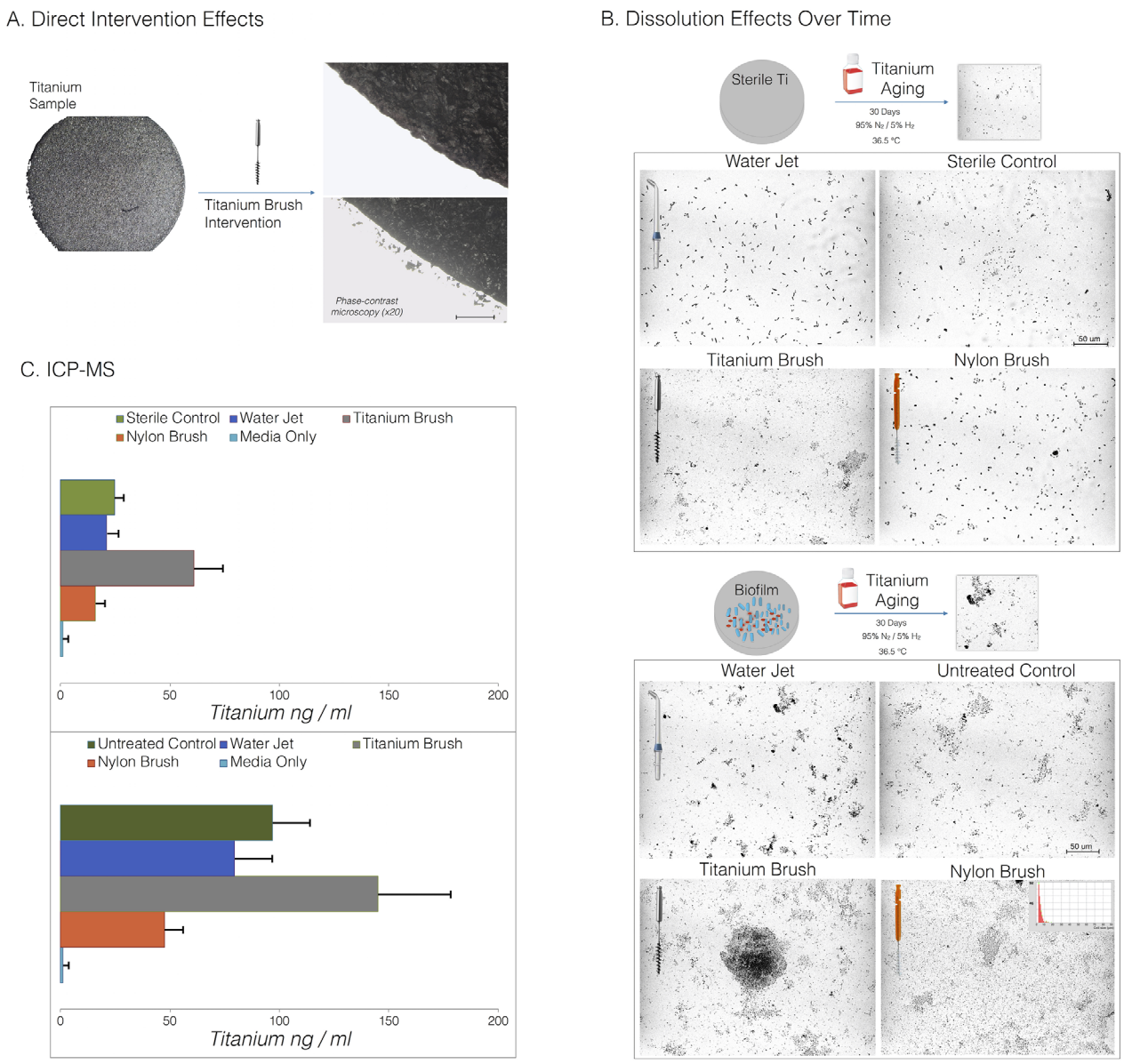

FIGURE 4 (**A**) Direct effect of titanium brush application on a test Ti surface; note the absence of particles adjacent to the implant surface and the large number of particle aggregates in the vicinity of the surface after 30 seconds of application. (**B**) Optical microscopy reveals Titanium Dissolution Products (TiDissPs) arising from all surfaces after 30 days of aerobic (top) or anaerobic (bottom) aging in simulated tissue fluids. A larger number of particles is noted in the "Ti Brush group" as compared to sterile controls under ambient conditions with particles tending to form clusters. Under anaerobic conditions that concentration of particles in the Ti Brush group increased by nearly four-fold; note the large particle aggregate in the center of the microphotograph. All test groups exhibited increased Ti under anaerobic conditions. (**C**) Inductively-coupled Mass Spectrometry (ICP-MS) was employed to assess Ti concentrations for each group (mean +/-SE). Both group and condition effects are noted with Ti brush and anaerobic aging exhibiting the largest dissolution potential, respectively

as compared to sterile control surfaces. Waterjet irrigation and Nylon brushes treated surfaces had the highest live cell counts among test interventions, but only WJ cell proliferation was significantly higher than controls ($P < 0.05$). Ti brush treated surfaces had significantly less viable cells proliferating as compared to sterile controls ($P < 0.05$) (Figure 5). CHX application demonstrated the lowest live cell counts and significantly more dead cells as compared to controls ($P < 0.05$), which is consistent with previous findings of chemotherapeutic agent adsorption with protracted cytotoxic effects on Ti surfaces.[8,32]

4 | DISCUSSION

Clinically, aggressive mechanical treatments are favored to remove peri-implant biofilms based on the empirical assumption that hardness determines effectiveness.

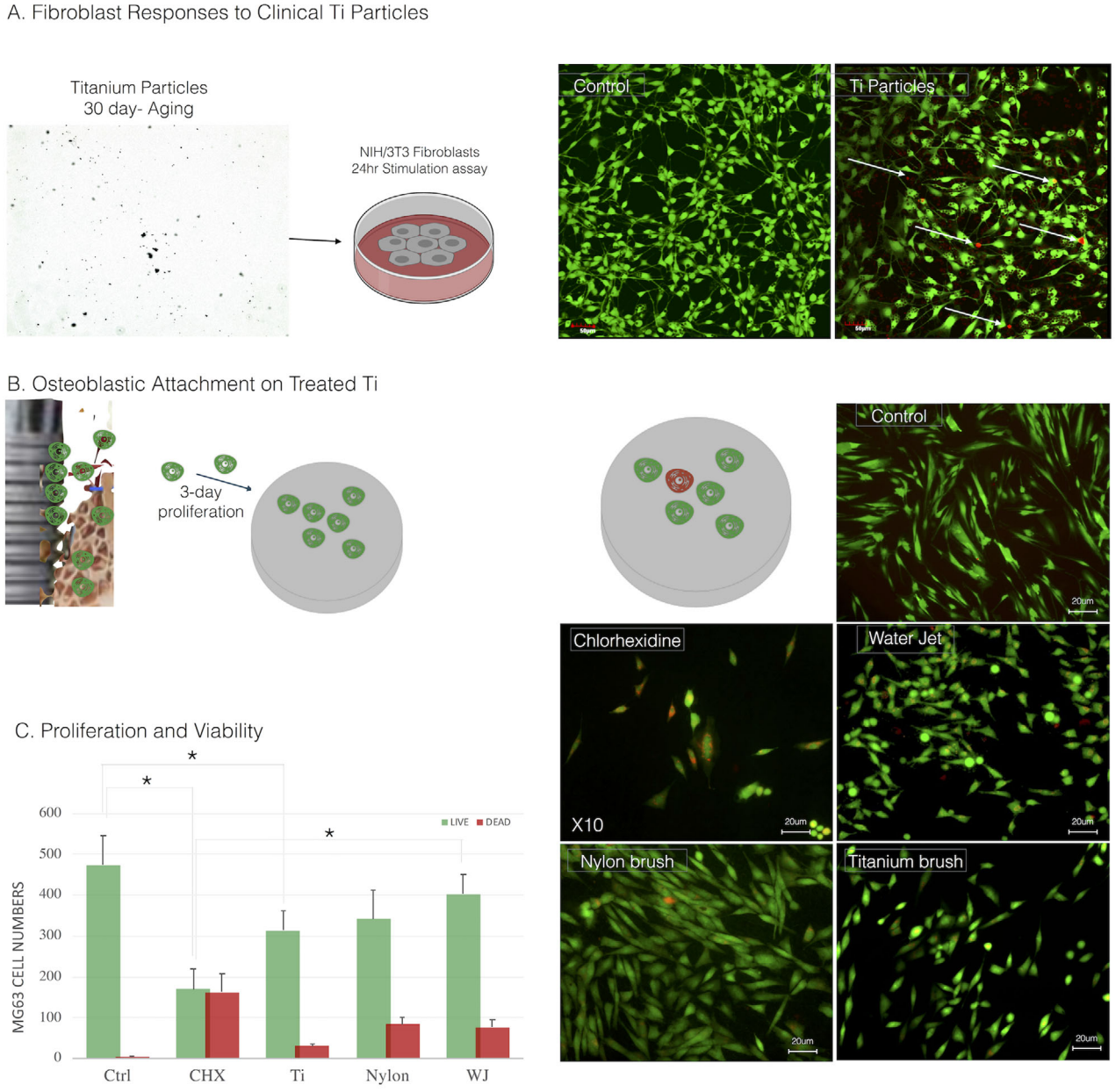

FIGURE 5 (**A**) Fibroblast cytotoxicity is apparent after 24 hours of stimulation with Titanium Dissolution Products (TiDissPs); arrows point to dead (red) cells. (**B**, **C**): in vitro re-osseointegration model assessing the proliferation of human osteoblastic cells on treated titanium surfaces after biofilm removal interventions. (Green: live cells; Red: dead cells)

Present results refute this assumption. Results showed that mechanical treatment of Ti implant surfaces with a cleaning aid of similar hardness to the implant, that is, Ti brush, led to surface alterations and increased Ti dissolution from the surface. These Ti surface changes were biologically adverse as they hindered the osteoconductivity of the treated surfaces. Notably, despite their aggressive mechanical effects, cleaning with Ti brushes did not improve antimicrobial efficacy. As determined by the partial removal of biofilm and the co-localization of biofilm removal areas with surface alterations under high magnification, it is plausible that mechanical aids with bristles engage the implant surface in a "patchy" fashion without rendering complete coverage. Nylon brushes had slightly better biofilm removal effects as compared to Ti brushes, which can be attributed to the increased elasticity of polymer bristles as compared to metal bristles, thus enabling the former to better conform on the implant surface. Conversely, water jet application led to near complete biofilm removal from the implant surface. Importantly, previous investigations for professional biofilm removal have focused on mechanical treatments, whereas the present study focused on the novel investigation of mechanical biofilm removal with interventions that minimize

abrasion to the implant surface under the hypothesis that these interventions would limit Ti dissolution.

Biofilm removal by WJ is supported by both in vitro and in vivo investigations. Gorur et al.[33] investigated the use of the water jet on teeth that were extracted because of severe periodontitis and found that the pulsating water jet irrigation removed over ninety nine percent of biofilm. In another study, a 3-day controlled intervention of either WJ irrigation or conventional string floss around implants exhibiting bleeding on probing (BOP) found a reduction frequency of 82% for the WJ versus 33% for the floss.[34] These results corroborate our findings of nearly total biofilm removal for the WJ only intervention in the present study. Importantly, the application of WJ did not lead to visible surface alterations or increased Ti dissolution as compared to controls, suggesting that among mechanical interventions factors such as mode of application of pressure, abrasiveness and hardness may be critical factors affecting surface effects on Ti. A strength of the present study is that the TiDissPs assessment occurred both under simulated anaerobic and aerobic conditions. Aerobic aging conditions are representative of shallow peri-implant sulci similar to healthy implants undergoing maintenance cleaning, whereas anaerobic conditions are representative of deep pockets with reduced partial oxygen pressure observed in peri-implantitis. Results showed that biofilm presence alone is adequate to increase TiDissPs by nearly four-fold in untreated versus sterile Ti surfaces, which is consistent with previous in vitro studies that used single species or polyculture Streptococcus biofilms.[35,36] Importantly, the Ti particles generated in the present model where primarily in the range of 1 to 10um diameter that has been shown to be most biologically reactive as compared to larger particle sizes.[12]

The consistent finding of vastly increased TiDissP concentrations in peri-implant tissues[15] and plaque biofilms[16] has recently led clinical researchers to challenge the removal of bacterial biofilms as being sufficient for the resolution of peri-implant inflammation.[21] To this extent, prior investigations of peri-implantitis as a multifactorial entity have established a strong association between TiDissPs and prevalent peri-implantitis.[16,22,37] Olmedo et al. were among the first to document the presence of TiDissPs in human peri-implant tissues via exfoliative cytology.[15] Specifically, Ti was observed both inside and outside epithelial cells and macrophages.[15] TiDissPs concentrations were increased under inflammatory conditions as compared to relative clinical health, which was suggestive of their role in peri-implant inflammation.[15] TiDissPs have been identified in peri-implant plaque,[16] and the host immune response has been shown to vary along with the amount of TiDissPs,[20] further highlighting the complex dynamics among biomaterial surfaces, oral biofilms and host immunity that define the peri-implant milieu. The long-term objective of this project is to determine the best method for treatment of biofilm-contaminated implant surfaces to allow for re-growth of osteoblastic cells on the treated surface, which is a proxy for clinical re-osseointegration. Results of osteoblastic proliferation in this work were suggestive of a biological effect of implant cleaning on preferentially grown tissue on the implant surface. Importantly, the present investigation highlights the need for multifaceted assessment of peri-implant treatment aids and agents through the spectrum of selective cytotoxicity and biomaterial degradation.

In summary, the significance of the findings of mechanical treatment triggering increased TiDissPS is paramount considering the striking peri-implantitis prevalence estimates in the USA and worldwide and the lack of an efficacious therapeutic approach. The translational value of the results presented in this submission is supported by a robust ex vivo model of ecological polymicrobial peri-implant biofilms. This ex vivo approach is based upon a previously described ecological peri-implant biofilm model grown on acid etched Ti discs to better recapitulate clinical conditions.[8,10] Using inoculation with clinical human peri-implant samples a robust and clinically relevant biofilm model is achieved, which is more than single—or three-species biofilm models that may be less resistant to interventions. Further, the biological translation of in vitro generated TiDissPs is supported by biological assays of key cell types participating in peri-implant soft and hard tissue homeostasis, that is, fibroblasts and osteoblasts. In regards to application in clinical practice, thus far most interventions recommended for implant surface cleaning have been developed based on simple antibacterial assays without specific attention to Ti surface properties and dissolution. The present work supports that the goal of peri-implantitis research should be to identify peri-implantitis treatments that effectively remove biofilms while maintaining the electrochemical properties of Ti to avoid accelerated Ti dissolution in peri-implant tissues and maintain Ti implants' biocompatibility. Ultimately, if the present in vitro and ex vivo results are validated in human studies then a major change on how implant surfaces are maintained and cleaned will be warranted in clinical practice. Prospective longitudinal clinical studies assessing Ti dissolution in vivo are critical for the advancement of management of peri-implant complications.

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CONFLICTS OF INTEREST

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AUTHOR CONTRIBUTIONS

Georgios A. Kotsakis designed the study. Georgios A. Kotsakis, Rachel Black, Jason Kum, Larissa Berbel, Ali Sadr, Ioannis Karoussis and Georgios A. Kotsakis performed data collection and data analysis. Georgios A. Kotsakis, Ioannis Karoussis, Mara Simopoulou and Diane Daubert have been involved in data interpretation, drafting the article and revising it critically. All authors have given final approval of the version to be published and all are responsible for the accuracy of the data presented.

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REFERENCES

1. Al-Kadhim KPM, Farnell D, Thomas D, Adams R, Claydon N. Surgical therapy for peri-implantitis management: a systematic review and meta-analysis. *Oral Surg*. 2018;11:200-212.
2. Schwarz F, Derks J, Monje A, Wang HL. Peri-implantitis. *J Periodontol*. 2018;89(Suppl 1):S267-S290.
3. Machtei E. Treatment alternatives to negotiate peri-implantitis. *Adv Med*. 2014;2014:13.
4. Ramanauskaite A, Becker K, Juodzbalys G, Schwarz F. Clinical outcomes following surgical treatment of peri-implantitis at grafted and non-grafted implant sites: a retrospective analysis. *Int J Implant Dent*. 2018;4:27.
5. Parlar A, Bosshardt DD, Cetiner D, et al. Effects of decontamination and implant surface characteristics on re-osseointegration following treatment of peri-implantitis. *Clin Oral Implants Res*. 2009;20:391-399.
6. Charalampakis G, Ramberg P, Dahlen G, Berglundh T, Abrahamsson I. Effect of cleansing of biofilm formed on titanium discs. *Clin Oral Implants Res*. 2015;26:931-936.
7. Schwarz F, Aoki A, Sculean A, Becker J. The impact of laser application on periodontal and peri-implant wound healing. *Periodontol 2000*. 2009;51:79-108.
8. Kotsakis GA, Lan C, Barbosa J, et al. Antimicrobial agents used in the treatment of peri-implantitis alter the physicochemistry and cytocompatibility of titanium surfaces. *J Periodontol*. 2016;87:809-819.
9. Ntrouka V, Hoogenkamp M, Zaura E, van der Weijden F. The effect of chemotherapeutic agents on titanium-adherent biofilms. *Clin Oral Implants Res*. 2011;22:1227-1234.
10. Karoussis IK, Kyriakidou K, Papaparaskevas J, Vrotsos IA, Simopoulou M, Kotsakis GA. Osteostimulative calcium phosphosilicate biomaterials partially restore the cytocompatibility of decontaminated titanium surfaces in a peri-implantitis model. *J Biomedical Mat Res Part B, Applied Biomaterials*. 2018;106:2645-2652.
11. Valderrama P, Wilson TG. Detoxification of implant surfaces affected by peri-implant disease: an overview of surgical methods. *Int J Dent*. 2013;2013:740680.
12. Eger M, Sterer N, Liron T, Kohavi D, Gabet Y. Scaling of titanium implants entrains inflammation-induced osteolysis. *Sci Rep*. 2017;7:39612.
13. Heyman O, Koren N, Mizraji G, et al. Impaired differentiation of langerhans cells in the murine oral epithelium adjacent to titanium dental implants. *Front Immunol*. 2018;9:1712.
14. Apaza-Bedoya K, Tarce M, Benfatti CAM, et al. Synergistic interactions between corrosion and wear at titanium-based dental implant connections: a scoping review. *J Periodontal Res*. 2017;52:946-954.
15. Olmedo DG, Nalli G, Verdú S, Paparella ML, Cabrini RL. Exfoliative cytology and titanium dental implants: a Pilot Study. *J Periodontol*. 2013;84:78-83.
16. Safioti LM, Kotsakis GA, Pozhitkov AE, Chung WO, Daubert DM. Increased levels of dissolved titanium are associated with peri-implantitis—a cross-sectional study. *J Periodontol*. 2017;88:436-442.
17. Islam AS, Beidelschies MA, Huml A, Greenfield EM. Titanium particles activate toll-like receptor 4 independently of lipid rafts in RAW264.7 murine macrophages. *J Orthop Res*. 2011;29:211-217.
18. Goncalves DM, de Liz R, Girard D. Activation of neutrophils by nanoparticles. *Sci World J*. 2011;11:1877-1885.
19. Pettersson M, Kelk P, Belibasakis GN, Bylund D, Molin Thoren M, Johansson A. Titanium ions form particles that activate and execute interleukin-1beta release from lipopolysaccharide-primed macrophages. *J Periodontal Res*. 2017;52:21-32.

20. Daubert DM, Pozhitkov AE, Safioti LM, Kotsakis GA. Association of global DNA methylation to titanium and peri-implantitis: a Case-Control Study. *JDR Clin Trans Res*. 2019;4:284-291.
21. Delgado-Ruiz R, Romanos G. Potential causes of titanium particle and ion release in implant dentistry: a systematic review. *Int J Mol Sci*. 2018;19:3585.
22. Rodrigues D, Valderrama P, Wilson T, et al. Titanium corrosion mechanisms in the oral environment: a retrieval study. *Materials*. 2013;6:5258-5274.
23. Fretwurst T, Nelson K, Tarnow DP, Wang HL, Giannobile WV. Is metal particle release associated with peri-implant bone destruction? An emerging concept. *J Dent Res*. 2018;97:259-265.
24. Daubert D, Pozhitkov A, McLean J, Kotsakis G. Titanium as a modifier of the peri-implant microbiome structure. *Clin Implant Dent Relat Res*. 2018;20:945-953.
25. Tian Y, He X, Torralba M, et al. Using DGGE profiling to develop a novel culture medium suitable for oral microbial communities. *Mol Oral Microbiol*. 2010;25:357-367.
26. Segata N, Waldron L, Ballarini A, Narasimhan V, Jousson O, Huttenhower C. Metagenomic microbial community profiling using unique clade-specific marker genes. *Nat Methods*. 2012;9:811-814.
27. Papathanasiou E, Finkelman M, Hanley J, Parashis AO. Prevalence, etiology and treatment of peri-implant mucositis and peri-Implantitis: a survey of periodontists in the United States. *J Periodontol*. 2016;87:493-501.
28. Kotsakis G, Goldie M, Konstantinidis I. Maintenance of patients with dental implants in contemporary dental practice. *Alpha Omegan*. 2015/2016;108:10-17.
29. Berbel LO, Banczek EDP, Karoussis IK, Kotsakis GA, Costa I. Determinants of corrosion resistance of Ti-6Al-4V alloy dental implants in an in vitro model of peri-implant inflammation. *PLoS One*. 2019;14:e0210530.
30. Att W, Hori N, Takeuchi M, et al. Time-dependent degradation of titanium osteoconductivity: an implication of biological aging of implant materials. *Biomaterials*. 2009;30:5352-5363.
31. Gemelli E. *Corrosion of Metallic Materials and Their Characterizatin*: LTC; 1st ed.; 2001.
32. Kozlovsky A, Artzi Z, Moses O, Kamin-Belsky N, Greenstein RB. Interaction of chlorhexidine with smooth and rough types of titanium surfaces. *J Periodontol*. 2006;77:1194-1200.
33. Gorur A, Lyle DM, Schaudinn C, Costerton JW. Biofilm removal with a dental water jet. *Compend Contin Educ Dent*. 2009;30(Spec No 1):1-6.
34. Magnuson B, Harsono M, Stark PC, Lyle D, Kugel G, Perry R. Comparison of the effect of two interdental cleaning devices around implants on the reduction of bleeding: a 30-day randomized clinical trial. *Compend Contin Educ Dent*. 2013;34(Spec No 8):2-7.
35. Souza JC, Henriques M, Oliveira R, Teughels W, Celis JP, Rocha LA. Do oral biofilms influence the wear and corrosion behavior of titanium?. *Biofouling*. 2010;26:471-478.
36. Siddiqui DA, Guida L, Sridhar S, Valderrama P, Wilson TG, Jr, Rodrigues DC. Evaluation of oral microbial corrosion on the surface degradation of dental implant materials. *J Periodontol*. 2019;90:72-81.
37. Fretwurst T, Buzanich G, Nahles S, Woelber JP, Riesemeier H, Nelson K. Metal elements in tissue with dental peri-implantitis: a pilot study. *Clin Oral Implants Res*. 2016;27:1178-1186.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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